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Expressed Peptide Assay for DNA Detection

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Abstract: Innovation in molecular diagnostics ultimately requires the conceptually distinct design of detection architectures. The diagnostic strategies reported thus far (planar/suspension arrays) suffer from either mass transport issues or limitations on the maximum number of targets that can be simultaneously detected. We report herein an expressed peptide assay scheme, by using nanoparticle probes, for detecting DNA hybridization events. The method exploits plasmid-encoded peptide tags as surrogate molecules for the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry identification of target DNA. The binding of target DNA is achieved through its recognition with a gold nanoparticle probe (functionalized with peptide-encoding plasmid and oligonucleotide complementary to part of the target sequence) and a microparticle probe (derivatized with oligonucleotide complementary to the rest of the target sequence). The magnetic separation of the three-component complex and expression of the peptide allows for the target identification by mass spectrometry. The detection of two DNA targets has been demonstrated through the selection of each individual tag for the respective target. Importantly, the modular nature of the probe design, by decoupling molecular binding events from peptide expression processes, should enable the ready extension of the methodology to the analysis of other species. An assay on a protein target has confirmed the efficacy of the conceptual framework proposed herein beyond the detection of DNA. The vast choice of mass tags offered by mass spectrometry provides significant advantages over previously documented assay systems.

Introduction

Advances in molecular level understanding of dynamic linkages and architectures of gene regulatory networks have led to a systems vision for health care and disease control.[1−3] This fundamental paradigm shift creates a pressing need for simultaneously assaying ever-increasing numbers of species from a small volume of samples.[4−6] For example, the detection of specific DNA sequences is important for the identification of disease states and infectious agents.[5] Extensive efforts have, therefore, been devoted to the development of various multiplexed diagnostic platforms, the dominant formats of which are planar arrays[4,7−9] and suspension (particle-based) arrays.[6,10−12] Each of these strategies has advantages and disadvantages, depending on the detection requirements. Planar array technologies offer ultra-high-density screening capabilities, but they can suffer from mass transport limitations in the molecular binding processes.[13] Suspension arrays partially overcome these limitations, but generally at the expense of reduced number of targets that can be multiplexed. It would be a significant advance if one could develop a diagnostic system with desired features from both perspectives. Herein, we report on an expressed peptide assay (EPA) strategy, by using nanoparticle (NP) probes, for detecting DNA hybridization events. The method exploits plasmid-encoded peptide tags as surrogate molecules for the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) identification of target DNA. In the proof-of-concept incarnation of this idea, our system is characterized by the utility of two types of particle probes (Figure 1). The first is a gold NP (AuNP) functionalized with peptide-encoding plasmid and oligonucleotide complementary to part of a target sequence. The second probe is a magnetic microparticle (MMP) derivatized with oligonucleotide complementary to the rest of the target sequence. The magnetic

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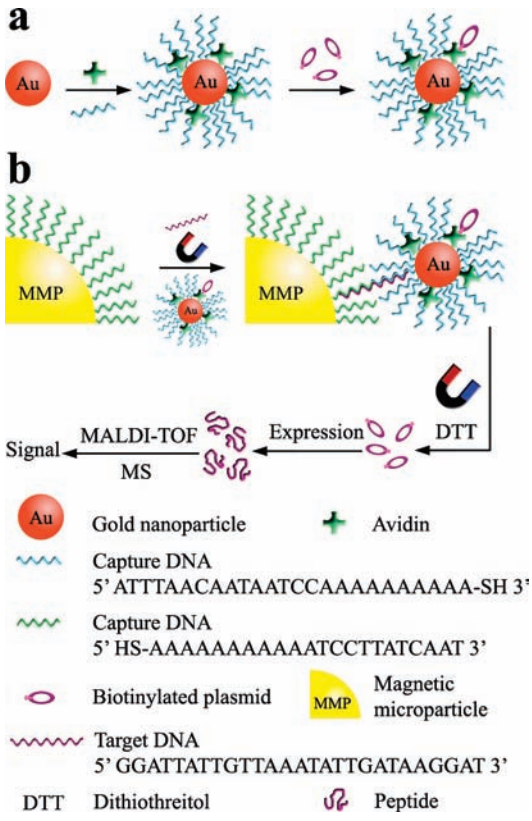

Figure 1. Schematic representation of the EPA method (structures are not drawn to scale). (a) AuNP probe design and fabrication. AuNP probe is designed in a modular fashion, with molecular recognition unit and signal generation unit independently attached to the surface. The modular approach ensures the broad target applicability of the AuNP probe. The fabrication of the AuNP probe is accomplished via initial functionalization of a citrate-stabilized AuNP with succinylated avidin and thiolated DNA and subsequent derivatization with biotinylated plasmid (psoralen−biotin adduct) through biotin−avidin interaction. (b) DNA detection through the MALDI-TOF MS identification of a surrogate peptide expressed by a plasmid. The EPA approach provides potential multiplexing capability in a solution format through the distinct choice of mass tags, without resorting to spatially resolved architectures. A typical EPA assay experiment starts with the hybridization of a DNA-loaded MMP probe with target DNA. The two-component complex is then concentrated through a magnetic separator. The isolated MMP−target hybrid is further reacted with a AuNP probe, and the three-component structure is again sequestered by the magnetic force. The plasmid is released from the AuNP probe via the addition of DTT, a thiol-containing small molecule with strong AuNP surface binding capability. The liberated plasmid is then subjected to *in vitro* expression. Target DNA is finally detected by the identification of a predesigned expressed peptide tag with MALDI-TOF MS.

separation of the sandwich structure formed by AuNP probe, MMP probe, and target DNA and expression of the peptide allows for the target identification by MALDI-TOF MS. The method reported herein presents us with a potential ability to perform multiplexed molecular diagnostics without resorting to the use of spatially resolved architectures. It provides an effective way to channel target species that are difficult or impossible to detect directly with MS (e.g., long-chain DNA) into arbitrarily selected, readable coding tags.

Results and Discussion

MS, by offering unsurpassed multichannel capability to measure a multitude of signatures concurrently, is the ultimate solution to multiplexing.[14−17] In addition, it is generally a more convenient tool compared with methods that rely on the utility of expensive fluorescent or radioactive labels. In spite of many advantages, the applicability of MS to molecules with inferior ionization efficiency and facile fragmentation[18] (e.g., DNA) is significantly restricted by virtue of the low signal intensity. We envisioned that the fabrication of NP probes functionalized with both the target-recognition unit and peptide-encoding plasmid would enable the translation of DNA hybridization events into peptide identification by MALDI-TOF MS. As the first test case, we provide a proof-of-concept demonstration on a known target DNA sequence (5′ GGATTATTGTTAAATATTGATAAGGAT 3′, **1**) associated with the anthrax lethal factor, the genomic information of which has been decoded.[19] The 27-base sequence, which is unique based on combinatorial probability consideration, has been employed elsewhere for demonstrating other detection schemes.[20−22] Prior documented examples rely on polymerase chain reaction (PCR) to create surrogate tags for the analysis of DNA.[23,24] In one case target detection is performed through the MALDI-TOF MS identification of expressed peptide.[24] However, significant drawbacks of this assay system include restriction of target to DNA, sensitivity to contamination due to the use of PCR, inflexibility in the choice of final peptide tag due to the inherent link between the target and peptide-encoding sequences, and low peptide yield due to the use of a linear template. Our NP-based diagnostic strategy decouples DNA hybridization events from peptide expression processes and, therefore, could be readily extended to the identification of other target species through the appropriate choice of target-recognition structures (e.g., antibodies for the identification of antigens).

The AuNP probe is prepared by a two-step procedure: initial functionalization of AuNPs with oligonucleotide and avidin, followed by derivatization with plasmid. Accordingly, AuNPs are first loaded with an oligonucleotide capture strand (5′ ATTTAACAATAATCCAAAAAAAAAA-SH 3′, **2**) and avidin by incubation in succinylated avidin and thiolated oligonucleotide, affording the AuNP preprobe. Biotinylated plasmid, fabricated by a photochemical reaction between biotin-psoralen and plasmid, is then coupled to the AuNP preprobe through a biotin−avidin interaction, generating the desired AuNP probe with capture oligonucleotide and plasmid. The plasmid, pET28a-ZHOU I (Supporting Information Figure S1), which encodes a tandem repeat sequence of a short peptide, was constructed by the insertion of a PCR-amplified tandem sequence into the *Nco*I and *Xho*I sites of pET28a. pET28a-ZHOU I was cloned in *E. coli* strain DH5α, and the sequence was verified by using

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primers adjacent to the two restriction sites. Analysis by gel electrophoresis is also consistent with the successful construction of the plasmid (Supporting Information Figure S2). Amine-functionalized MMPs are modified with a second oligonucleotide capture strand (5′ HS-AAAAAAAAAAATCCTTAT-CAAT 3′, **3**) and a passivation strand (5′ HS-AAAAAAAAAA 3′) through the coupling reaction with a heterobifunctional cross-linker molecule, succinimidyl-4-(*p*-maleimidophenyl)butyrate (SMPB),[25] affording the MMP probe. The detection of target DNA through the EPA method involves the capture of target DNA and the AuNP probe with the MMP probe, the release of the plasmid, the expression of the peptide, and the MALDI-TOF MS characterization. Briefly, the MMP probe is first hybridized with target DNA and the AuNP probe in a two-step fashion. The plasmid on the AuNP probe is then released with DTT, a molecule with two thiol groups and strong surface-binding capability. *In vitro* expression of the plasmid and desalting of the resulting peptide enables the detection of target DNA through the MALDI-TOF MS identification of a surrogate molecule. The application of AuNP probes in our EPA system offers several advantages over conventional assays: (1) The separation of plasmid attachment and target-recognition unit derivatization would enable the chemistry to be generically applicable to other target structures. If AuNPs are not used as the carrier, specific chemistry has to be worked out for the coupling of each individual target recognition unit and plasmid, thus restricting the extensibility of the probe fabrication process. (2) Complicated coupling and purification steps generally required for the fabrication of bioconjugate probes are avoided. The AuNP-based method involves only derivatization of each molecular component onto the surface and a subsequent centrifugation step at predefined speed, thus enabling the facile preparation of any desired probes. (3) The amplification can be conveniently achieved in part by loading a high percentage of peptide-encoding plasmids. One could in principle take advantage of larger-sized AuNPs as the carrier and ensure the transduction of one binding event into the release of multiple plasmids. (4) In principle, superior selectivity could be accomplished by virtue of the unusually sharp melting profiles associated with AuNP probes.[20] The collective properties exhibited by functionalized AuNPs, by virtue of the loading of multiple ligands, are expected to be a generic phenomenon for any molecular binding events.

The as-prepared AuNP preprobe exhibits an intense red color similar to citrate-capped AuNPs (Supporting Information Figure S3), which is associated with the surface plasmon resonance (SPR) band at around 520 nm (Supporting Information Figure S4). The apparent decrease of the SPR band intensity on functionalization is a reflection of decreased particle concentration. The successful derivatization of avidin was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 2). Loading of the capture oligonucleotide and retention of the hybridization capability on the AuNP preprobe was demonstrated by a sandwich assay with target DNA and MMP probe. This set of experiments is essential to ruling out hybridization interference from the derivatization as observed in other systems.[26] Thus, in the presence of target DNA, in a manner analogous to pure oligonucleotide-modified AuNP probe, the AuNP preprobe and MMP probe form a

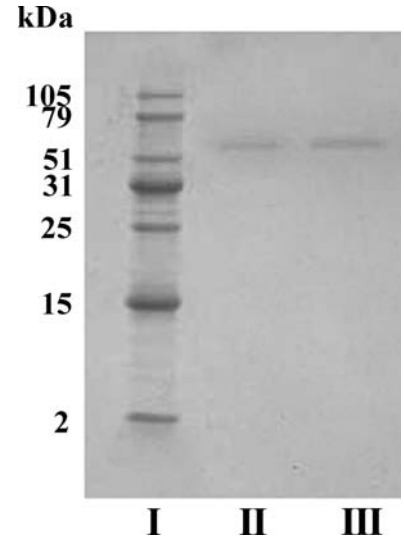

Figure 2. Gel electrophoresis showing the loading of succinylated avidin onto the AuNP preprobe (with capture strand **2** incorporated). SDS-PAGE (12%) diagram of the AuNP preprobe. Lane I: Protein marker. Lane II: AuNP preprobe (the surface-immobilized succinylated avidin was dissociated from the AuNP preprobe through boiling at 100 °C for 5 min before SDS-PAGE was performed). Lane III: Succinylated avidin standard. An identical protein was used as the standard (lane III) to confirm the successful modification of the AuNP preprobe with succinylated avidin.

complex, which can be drawn to the wall of a reaction vessel by the application of a magnetic field (Supporting Information Figure S5). Consistent with this, a control sample without target DNA leaves the AuNP preprobe detached from the MMP probe. It should be noted that the red-colored solution background from the AuNP preprobe could cause subtle visual effects on the color of the MMP probe, even though no adsorption actually takes place (Supporting Information Figures S6 and S7). Characterization with scanning electron microscopy (SEM) provides direct evidence for the formation of a complex structure from the AuNP preprobe and MMP probe only in the presence of target DNA (Supporting Information Figure S8).

Significantly, the AuNP probe also displays a red, although less intense color after the two rounds of centrifugation processes, consistent with the existence of individually dispersed AuNPs (Supporting Information Figure S3). The incorporation of biotinylated plasmid into the AuNP probe was supported by a gel electrophoresis experiment (Figure 3). The AuNP probe retains the hybridization competence of the oligonucleotide capture strand, as substantiated by the ability to magnetically separate the sandwich complex formed with the MMP probe and target DNA (Figure 4). The uniform distribution of AuNPs on MMP surfaces demonstrates the quality of the fabricated probes (Figure 5). Macroscopically observable loss of red color associated with the AuNP probe occurs only at target concentrations higher than the nanomolar range (Supporting Information Figure S9), typical for colorimetric assays.[27] The number of plasmid, avidin, and oligonucleotide molecules on a single AuNP probe particle was determined to be ∼1, 4, and 30, respectively (Supporting Information Figures S4 and S10−S12). Although on average there exist three free avidin sites on a AuNP probe, the scrambling between different probes in the multiplexed analysis settings is highly unlikely based on the following grounds: (1) The interaction between avidin and biotin

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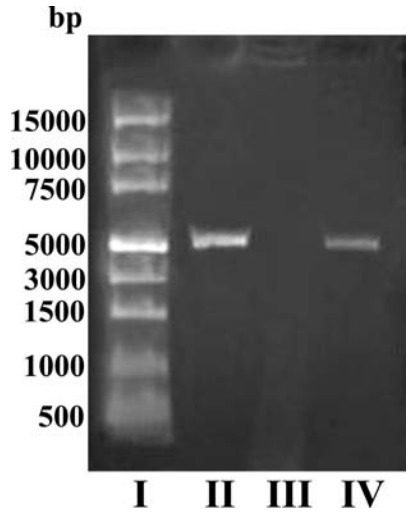

Figure 3. Gel electrophoresis showing the loading of biotinylated plasmid onto the AuNP probe (with capture strand **2** and biotinylated pET28a-ZHOU I incorporated). Agarose (1.0%) gel electrophoresis diagram of the AuNP probe. Lane I: DNA marker. Lane II: AuNP probe (the surface-immobilized biotinylated plasmid was dissociated from the AuNP probe through boiling at 90 °C for 20 min before gel electrophoresis was performed). Lane III: Pure oligonucleotide (**2**)-modified AuNPs after incubation with the biotinylated plasmid. Lane IV: pET28a.

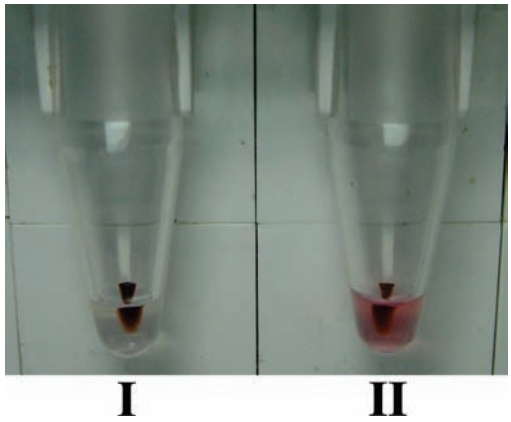

Figure 4. Retention of the hybridization competence of oligonucleotide capture strands on the AuNP probe (with capture strand **2** and biotinylated pET28a-ZHOU I incorporated) and the MMP probe (with capture strand **3** incorporated). I: Macroscopic image of a solution mixture comprising AuNP probe, MMP probe, and target DNA **1** on a magnetic separator. II: Macroscopic image of a solution mixture comprising AuNP probe and MMP probe on a magnetic separator.

is extremely strong, and (2) exchange of molecules anchored on a NP surface is extremely difficult (excess free avidin molecule and biotinylated plasmid have been removed during the probe preparation step). Considering the quadratic relationship between the surface area and diameter of AuNP, the loading of plasmid could be increased through the use of larger-sized particles. The absence of nonspecific binding between the plasmid and MMP probe was confirmed through gel electrophoresis. Indeed, no plasmid was observed on a magnetically isolated component in a solution containing only the MMP and AuNP probes (Supporting Information Figures S13 and S14). As expected, at a high initial target concentration (8 μM), only a small fraction (0.39%) of the DNA molecules can be captured (Supporting Information Figure S15), reflecting the limited amount of oligonucleotides on MMP that is available for hybridization. Under these experimental conditions, the fraction

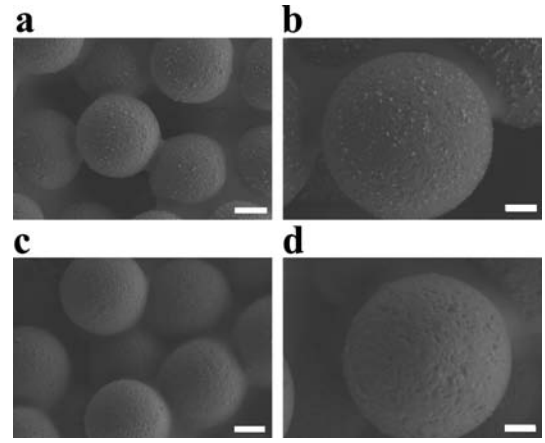

Figure 5. Microscopic confirmation of the formation of complex structures from the AuNP probe (with capture strand **2** and biotinylated pET28a-ZHOU I incorporated), the MMP probe (with capture strand **3** incorporated), and target DNA **1**. (a) Low-magnification SEM image of a sample generated from the AuNP probe, MMP probe, and target DNA (scale bar: 1 μm). (b) High-magnification SEM image of a sample generated from the AuNP probe, MMP probe, and target DNA (scale bar: 0.5 μm). (c) Low-magnification SEM image of a sample generated from the AuNP probe and MMP probe (scale bar: 1 μm). (d) High-magnification SEM image of a sample generated from the AuNP probe and MMP probe (scale bar: 0.5 μm).

of captured plasmid is 61% (Supporting Information Figure S14). To better understand the authentic hybridization efficiency associated with the system, a lower initial amount of target should be employed. Indeed, direct release of the target DNA indicates high hybridization efficiencies (Supporting Information Figures S16 and S17) at these concentrations (92% and 93% for 1 and 3 nM, respectively).

The ability to produce the peptide from pET28a-ZHOU I for target DNA detection at micromolar concentration was confirmed initially with SDS-PAGE (Supporting Information Figure S18). To this end, the plasmid was first released with dithiothreitol (DTT) from the complex structure generated by the AuNP probe, the MMP probe, and target DNA. The percentage of target DNA and plasmid obtained using this protocol was determined to be 77% and 61% (Supporting Information Figures S19 and S20), respectively. Therefore, the number of plasmid molecules on a AuNP probe particle, which could be maximized by using larger-sized particles, plays a crucial role in the first-tiered signal amplification. The released plasmid was purified through a 10 kD centrifuge filter device and subjected to *in vitro* expression in the *E. coli* T7 S30 extract. The tandem repeat peptide can be cleaved into the desired tag peptide (GLERDVHEVYRN, **4**) by hydroxylamine, which acts preferentially on the N−G linkage.[28] The tag peptide was designed by modifying the structure of an antimicrobial peptide (RLCRIVVIRVCR)[29] based on the following considerations: (1) The oligonucleotide that encodes the tandem repeat peptide should not contain commonly used restriction sites in molecular biology so that it can be inserted into other types of plasmids if desired; (2) the expression level should be maximized through the accommodation of a balanced number of acidic, basic, and hydrophobic amino acids; (3) no cysteine residue should be incorporated into the peptide because the formation of disulfide bonds is undesired for MALDI-TOF MS analysis; and (4) the molecular weight of the tag peptide should be between 1000 and 2000, a range

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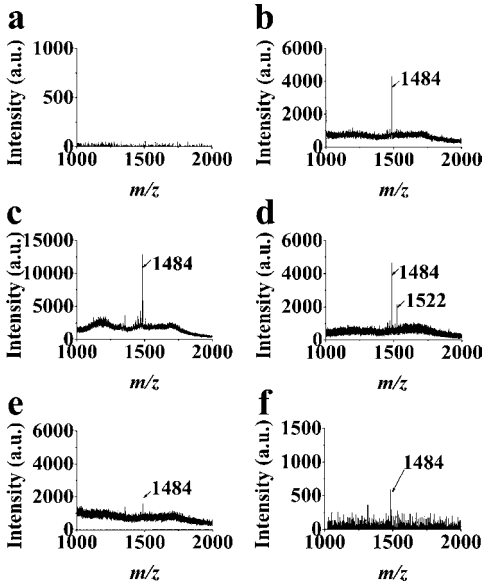

Figure 6. Single-target (**1**) DNA detection via the MALDI-TOF MS identification of a peptide (**4**) encoded by pET28a-ZHOU I. (a) MALDI-TOF MS spectrum in the absence of target. (b) MALDI-TOF MS spectrum in the presence of a 40 μM target (1484, $[\mathbf{4} + H]^+$). (c) MALDI-TOF MS spectrum of an independently synthesized, authentic peptide with the sequence GLERDVHEVYRN. (d) MALDI-TOF MS spectrum in the presence of a 40 nM target (1522, $[\mathbf{4} + K]^+$). (e) MALDI-TOF MS spectrum in the presence of a 40 pM target. (f) MALDI-TOF MS spectrum in the presence of a 4 pM target. The noise in the spectrum is generally considered chemical in nature,[30] which could be derived from ions of fragments and clusters of analyte and matrix.

ideally suited for MALDI-TOF MS detection due to the high signal intensity and lack of interference from the externally added matrix.

With all the probes in hand, we next examined the feasibility of using MALDI-TOF MS for the identification of a surrogate tag peptide as a means for DNA detection. Before subjecting to MS analysis, the hydroxylamine-cleaved peptide was concentrated, purified, and desalted using ZipTip, a type of pipet tip with chromatography media at its end. α-Cyano-4-hydroxycinnamic acid (HCCA), a widely used matrix for peptide mapping, was used in all experiments. Indeed, in the presence of micromolar and nanomolar target DNA, an intense peak at 1484 associated with the tag peptide could be observed (Figure 6). Confirmation of the assignment was provided through the MALDI-TOF MS analysis of an independently synthesized peptide with the identical sequence. In addition to the proton adduct ion, other species, such as potassium adduct ion, could be occasionally identified. No tag peptide peak was observed in the absence of target DNA, indicating the lack of false positives. The signal amplification at three levels ensures a high sensitivity achievable by the EPA strategy: multiple plasmids associated with each DNA hybridization event (through the use of larger-sized AuNPs), multiple mRNA transcripts from each plasmid template, and multiple peptide molecules from each mRNA transcript. We could detect target DNA at a concentration as low as 4 pM, a biologically relevant value (Supporting Information Figure S21), which translates into an absolute amount of 40 attomoles in a 10 μL solution under unoptimized conditions. Given the quantitative capability demonstrated for MALDI-TOF MS,[31] quantification of target DNA or other

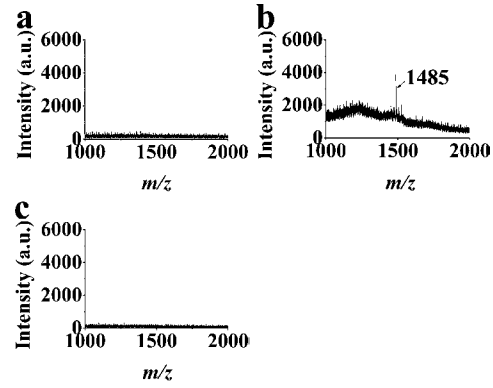

Figure 7. Long-chain single-target (**5**) DNA detection via the MALDI-TOF MS identification of a peptide (**4**) encoded by pET28a-ZHOU I. (a) MALDI-TOF MS spectrum in the absence of target. (b) MALDI-TOF MS spectrum in the presence of 10 μM target DNA **5** (1485, $[\mathbf{4} + H]^+$; a slight variation of the peak position could be observed). (c) MALDI-TOF MS spectrum in the presence of 10 μM control DNA **6**.

species with our EPA strategy is expected to be feasible. For example, an isotope-labeled or structurally closely related peptide could be used as the internal standard for the relative quantification of an EPA-derived peptide. Optimization of the probe structures (e.g., size of AuNPs, type and sequence of plasmid) and preparation conditions (e.g., percentage of plasmid loaded on AuNPs) is expected to lead to a further increase of the assay sensitivity. Direct characterization of DNA with MALDI-TOF MS indicates that a target strand of 40 μM could be reproducibly identified under the experimental conditions employ herein (Supporting Information Figures S22 and S23). Thus, our EPA scheme provides at least several orders of magnitude sensitivity advantage over the direct analysis protocol. The ability to detect a long-chain DNA target was demonstrated with a model sequence (5′ AAAAAAAAAAAAAAAGGATTATTGTTAAAATATTGATAAAG-GATAAAAAAAAAAAAAAA 3′, **5**), and non-complementary DNA (5′ AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 3′, **6**) did not interfere with the assay (Supporting Information Figure S24 and Figure 7). The method demonstrated herein could be developed into a general tool for the MS analysis of biologically important structures, interactions, and functions.[32] The EPA strategy should enable the examination of mass spectrometrically inaccessible molecules (e.g., because of low abundance or structural factors) through the signal amplification process.

We have demonstrated previously a monolayer-barcoded NP strategy, by using small organic molecules as tag moieties, for the MALDI-TOF MS detection of DNA.[14] The variety of tags accessible from the ethylene glycol framework of that system is limited, placing constraints on the maximum number of multiplexed targets. Attempts at employing small peptides as tags proved unsuccessful due to the facile aggregation of AuNPs induced by this type of structure.[33] The potential disadvantage of our enabling technology lies in the extra steps that are used to obtain the surrogate peptide. However, the extra steps can be performed routinely, and the EPA strategy has provided us

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with a capability to expand the peptide tag repertoire and therefore shows promise in paralleled multiplexed analysis in a solution format with reasonable cost.

The use of negatively charged, oligonucleotide-based structures (target-recognition oligonucleotides and plasmids) in AuNP probes creates an ideal, densely packed surface environment for the elimination of cross reactivity and nonspecific binding, issues that are challenging to address in other assay systems applied in more complex settings.[25] We have therefore next demonstrated the ability to carry out a DNA hybridization assay for two target strands simultaneously. Although a distinct peptide tag could be acquired through the variation of a tandem repeat sequence inserted in pET28a-ZHOU I, we opted for a recently developed N^{pro} fusion technology[34] and constructed a completely different plasmid (pET30a-EDDIE-ZHOU II) (Supporting Information Figure S25). This is to demonstrate that a wide variety of plasmids can be integrated into the AuNP probes of our EPA system, and molecular weights of the mass tags can be extended beyond the 1000−2000 range. To achieve this, a PCR-amplified sequence encoding a CM4 peptide (RWKIFKKIEKVGQNIRDGIVKAGPAVAVVGQAATI, **7**)[35] was inserted into the *Spe*I and *Sal*I sites of pET30a-EDDIE. The successful construction of the plasmid was verified by both gel electrophoresis (Supporting Information Figure S26) and DNA sequencing. The autoproteolytic function of N^{pro} allows the release of a fusion partner and production of peptide with an authentic N terminus. The target DNA strand (5′ TATGTTAGTATGATATAGGAATAGTTA 3′, **8**) to be detected has the same base composition as **1** but is arranged in a different order. The selection of this DNA sequence is to demonstrate that, through the appropriate choice of peptide tag, DNA targets of the same molecular weight could be differentiated by MALDI-TOF MS. The AuNP probe was fabricated by an identical procedure to that described earlier, except being loaded with pET30a-EDDIE-ZHOU II and a different capture strand (5′ CATACTAACATAAAAAAAAAAA-SH 3′, **9**). Likewise, the MMP probe was prepared with a capture strand (5′ HS-AAAAAAAAAATAACTATTCCTATAT 3′, **10**) that can form a sandwich structure with the target DNA and AuNP probe. The ability to perform two-target analysis in a solution format is indeed a desirable feature of our diagnostic platform and a significant advance over many existing detection systems. For example, although one can use macroscopically observable magnetic separation of the red AuNP probe by the MMP probe for the identification of a single target, it is impossible to use the same format to distinguish multiple targets simultaneously (Supporting Information Figures S27−S29). In contrast, through MS, we could perfectly differentiate and detect target strands **1** and **8** concurrently by virtue of the presence and absence of respective peptide indicator peaks, even down at the picomolar concentration range (Figures 8−10). The above data offer unambiguous evidence that the EPA method is an ideal tool for the detection of DNA hybridization events. Optimization of the protocol for distinct types of target species will enable the application of our assay scheme in real biological settings.

The applicability of our EPA system beyond the detection of DNA is validated through a proof-of-concept demonstration

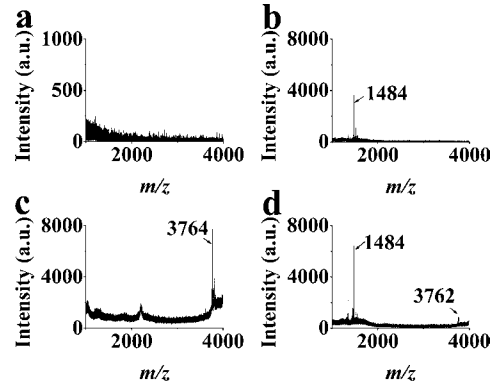

Figure 8. Two-target (**1** and **8**) DNA detection via the MALDI-TOF MS identification of peptides (**4** and **7**) encoded by pET28a-ZHOU I and pET30a-EDDIE-ZHOU II, respectively. (a) MALDI-TOF MS spectrum in the absence of target. (b) MALDI-TOF MS spectrum in the presence of 40 μM target **1** (1484, $[\mathbf{4} + H]^+$). (c) MALDI-TOF MS spectrum in the presence of 40 μM target **8** (3764, $[\mathbf{7} + H]^+$). (d) MALDI-TOF MS spectrum in the presence of 40 μM each of target **1** and target **8** (3762, $[\mathbf{7} + H]^+$; a slight variation of the peak position could be observed).

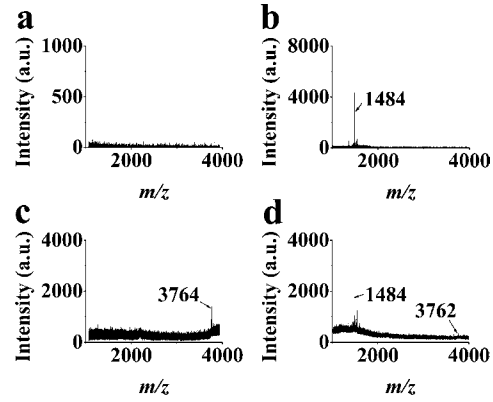

Figure 9. Two-target (**1** and **8**) DNA detection via the MALDI-TOF MS identification of peptides (**4** and **7**) encoded by pET28a-ZHOU I and pET30a-EDDIE-ZHOU II, respectively. (a) MALDI-TOF MS spectrum in the absence of target. (b) MALDI-TOF MS spectrum in the presence of 40 nM target **1** (1484, $[\mathbf{4} + H]^+$). (c) MALDI-TOF MS spectrum in the presence of 40 nM target **8** (3764, $[\mathbf{7} + H]^+$). (d) MALDI-TOF MS spectrum in the presence of 40 nM each of target **1** and target **8** (3762, $[\mathbf{7} + H]^+$; a slight variation of the peak position could be observed).

on a protein molecule. BAFF (B cell activating factor of the TNF family) is selected as the test analyte because of its importance in the development and function of B cells.[36] The modular nature of the probe design indeed allowed the facile preparation of the AuNP probe for this target species (Figures S30 and S31). Gel electrophoresis unambiguously confirmed the presence of succinylated avidin, anti-BAFF antibody, and biotinylated plasmid (pET28a-ZHOU I) on the probe surface and permitted quantification of the molecules (Figures S32 and S33). The AuNP probe, together with BAFF target and MMP probe (with anti-BAFF antibody incorporated), could effectively form a magnetically isolable sandwich complex (Figure S34) through the antigen−antibody interaction. Significantly, subsequent expression of the peptide provides an EPA signal readout for protein down to the 1 pM level (Figure 11), much more sensitive than the direct assay scheme (Figure S35).

Taken together, the EPA assay strategy, built upon a cheap source of biological information-encoding unit, provides an

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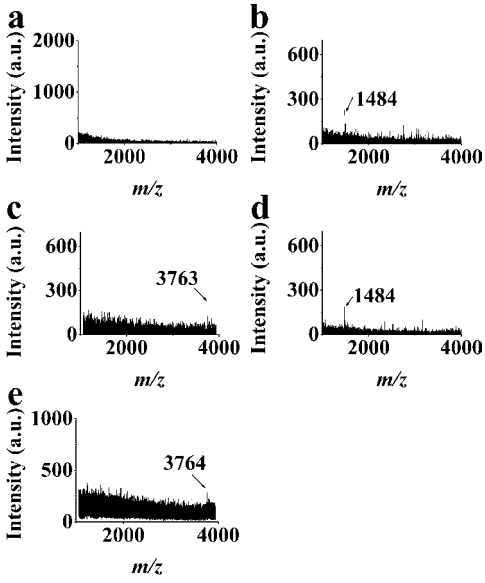

Figure 10. Two-target (**1** and **8**) DNA detection via the MALDI-TOF MS identification of peptides (**4** and **7**) encoded by pET28a-ZHOU I and pET30a-EDDIE-ZHOU II, respectively. (a) MALDI-TOF MS spectrum in the absence of target. (b) MALDI-TOF MS spectrum in the presence of 40 pM target **1** (1484, $[\mathbf{4} + H]^+$). (c) MALDI-TOF MS spectrum in the presence of 40 pM target **8** (3763, $[\mathbf{7} + H]^+$). (d) MALDI-TOF MS spectrum in the presence of 40 pM each of target **1** and target **8**. (e) MALDI-TOF MS spectrum in the presence of 40 pM each of target **1** and target **8** (3764, $[\mathbf{7} + H]^+$; a slight variation of the peak position could be observed). In the presence of 40 pM each of target **1** and target **8**, the peaks of $[\mathbf{4} + H]^+$ and $[\mathbf{7} + H]^+$ could not be observed simultaneously in one laser-sampled spot. Instead, they appeared separately at different locations. However, the identification of both peaks indicates the presence of two targets.

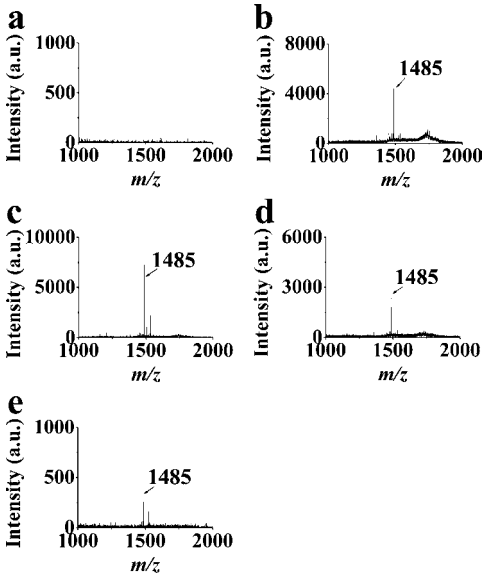

Figure 11. Protein (BAFF) detection via the MALDI-TOF MS identification of a peptide (**4**) encoded by pET28a-ZHOU I. (a) MALDI-TOF MS spectrum in the absence of target. (b) MALDI-TOF MS spectrum in the presence of 1 μM BAFF (1485, $[\mathbf{4} + H]^+$; a slight variation of the peak position could be observed). (c) MALDI-TOF MS spectrum of an independently synthesized, authentic peptide with the sequence GLERDVHEVYRN. (d) MALDI-TOF MS spectrum in the presence of a 1 nM target. (e) MALDI-TOF MS spectrum in the presence of a 1 pM target.

elegant way to unravel complex molecular interactions. The vast choice of mass tags offered by MS provides significant advantages over previously documented assay systems, such as expression immunoassay.[37] The luminescent signal readout scheme restricts the method to a limited number of individually discernible labels. Further, the requisite correct folding of expressed product into a functional enzyme is more technically demanding than the nonfunctional peptide used in our EPA strategy and, therefore, more prone to false negatives. Also, in principle, a high yield is expected for the low molecular weight peptide. Moreover, plasmids are superior over linear strands in several aspects, including readiness for phenotypic selection, ease of amplification, and stability against nuclease activity, thus facilitating the fabrication of large quantities of probes and administration of the EPA tool in complex environments.

Conclusion

In summary, a DNA detection strategy based on the MALDI-TOF MS identification of a plasmid-expressed surrogate peptide is reported and potentially allows for high throughput and high target density. Pairing oligonucleotide strands with individual tags expressed from an essentially unlimited array of synthetic plasmids shows promise in multiplexed detection of DNA in a solution format. The modular nature of the probe design, by decoupling molecular binding events from peptide expression processes, provides a sound basis for the extension of the methodology to the analysis of other targets of interest.

Experimental Section

Materials. The vectors pET28a and pET30a were purchased from Novagen. Succinylated avidin, ATP, dNTP, and $MgCl_2$ were obtained from Sigma-Aldrich. The peptone and yeast extract were from OXOID. The ligase was from Takara. The restriction endonucleases and Pfu polymerase were from New England Biolabs. The DNA marker was from Transgene. The prestained protein marker was from Generay. The SDS-gradient gel was from Jinsite. SMPB was from Pierce. Psoralen-biotin was from Ambion. *E. coli* T7 S30 extract was from Promega. The thiolated DNA, target DNA, primers, and amine-terminated Dynalbead M270 MMP were from Invitrogen. Ammonium acetate and hydroxylamine were from Alfa Aesar. Tween 20 was from BBI. ZipTipC18 pipet tips and the 10 kD centrifuge filter device were from Millipore. The Plasmid Purification Kit was from Axygen.

Measurement. The MALDI-TOF MS measurement was performed on a Bruker Daltonics Autoflex II. The MS data were acquired in the reflectron mode unless otherwise noted.

Construction of pET28a-ZHOU I. A tandem repeat sequence (5′ AACGGACTAGAAAGGGATGTGCACGAAGTATACAGGAACGGACTAGAAAGGGATGTGCACGAAGTATACAGGAACGGACTAGAAAGGGATGTGCACGAAGTATACAGGAACGGACTAGAAAGGGATGTGCACGAAGTATACAGG 3′) was amplified by PCR (forward: 5′ ATATCCATGGGCAACGGACTAGAAAG GGAT 3′; reverse: 5′ ATAGCTCGAGGATTCCGTTCCTGTATACTTC 3′; the underlined nucleotides are *Nco*I and *Xho*I recognition sites, respectively) for 30 cycles of 30 s at 95 °C, 30 s at 55 °C, and 1 min at 72 °C, and then a final extension at 72 °C for 10 min was performed. After digestion at *Nco*I and *Xho*I sites, the above genes were inserted into the pET28a vector and cloned in *E. coli* strain DH5α. The entire DNA sequence of the inserted portion was verified on both strands using sequencing primers adjacent to the *Nco*I and *Xho*I sites of pET28a. The recombinant plasmid is designated as pET28a-ZHOU I.

Construction of pET30a-EDDIE-ZHOU II. An antibacterial peptide gene (5′ CGTTGGAAGATCT TTAAGAAAATCGAAAAGGTTGGTCAGAACATCCGTGACGGTATCGT-

(37) Christopoulos, T. K.; Chiu, N. H. L. *Anal. Chem.* **1995**, *67*, 4290–4294.

T A A G G C T G G T C C G G C T G T T G C T G T T G G T G - GCCAGGCTGCCACTATC 3′) was amplified by PCR (forward: 5′ ATATACTAGTTGCCGTTGGAAGTTTAAG 3′; reverse: 5′ ATATGTCGACTTATTAGATAGTGGCAGC 3′; the underlined nucleotides are *Spe*I and *Sal*I recognition sites, respectively) for 35 cycles of 30 s at 94 °C, 1 min at 54 °C, and 1 min at 72 °C. The above gene was subcloned through *Spe*I and *Sal*I restriction sites into pET30a-EDDIE, a plasmid kindly provided by Dr. Bernhard Auer. The subcloned sequence was verified by DNA sequencing. The recombinant plasmid is designated as pET30a-EDDIE-ZHOU II.

Biotinylation of Plasmid. The two plasmids were transfected into *E. coli* strain DH5α and amplified in LB culture media. A substantial amount of plasmids was obtained by the Plasmid Purification Kit from Axygen and stored in TE buffer at −20 °C. The biotinylation of plasmid was carried out by following the manufacturer's protocol (BrightStar Psoralen-Biotin Kit, Ambion). The resulting biotinylated plasmid was stored in TE buffer at −20 °C. The biotinylation was indirectly verified by agarose gel electrophoresis.

Preparation of AuNP Preprobe. Prior to functionalization, the pH of AuNPs was adjusted to 9.0 using 0.2 M NaOH. First, the 13 nm AuNPs (1 mL) were functionalized with avidin by adding 10 μL of succinylated avidin (1 mg/mL), incubating the mixture solution for 30 min at 25 °C under vortex. Thiolated DNA was then added to the above solution (final oligonucleotide concentration: 15−20 μM). The reaction was allowed to proceed under gentle shaking conditions for 16 h at 25 °C. The solution was then brought to 0.1 M NaCl, 10 mM phosphate buffer (pH 7.0) in a stepwise manner with 0.3 M NaCl, 30 mM phosphate buffer (pH 7.0) and allowed to age for 12 h at 25 °C. Excess succinylated avidin and thiolated oligonucleotide were removed by repeated centrifugation at 15 000 rpm for 20 min (4 °C) and washing two times with the assay buffer (0.2 M NaCl, 10 mM phosphate buffer, 0.1% Tween 20, pH 7.2). The AuNP preprobe was finally redispersed in the assay buffer and stored at 4 °C prior to use.

Preparation of AuNP Probe. The AuNP preprobe (900 μL) was mixed with biotinylated plasmid (20 μg), and the reaction was allowed to proceed for 1 h at 37 °C. The functionalized AuNPs were purified by repeated centrifugation at 13 000 rpm for 20 min (4 °C) and washing two times with the assay buffer. The AuNP probe was finally redispersed in the assay buffer and stored at 4 °C prior to use.

Preparation of MMP Probe. The fabrication of the MMP probe was carried out by following a literature protocol.[22]

Target DNA Detection. The assay was initiated by mixing assay buffer (20 μL), MMP multiplexing solution (20 μL, 4.5 mg/mL), and the appropriately mixed target solution (10 μL) in a 1.5 mL Eppendorf tube. The system was heated at 45 °C for 30 min, followed by incubation at 25 °C for 3 h under constant vortex to allow hybridization between the MMP probes and target DNA strands. Then the reaction tube was placed on a magnetic separator and left standing for a few minutes, and the MMP−target complexes were washed twice with the assay buffer (200 μL). AuNP probe solution composed of two AuNP probes (AuNP multiplexing solution) was prepared by diluting equal volumes of each AuNP probe in the assay buffer to a final total AuNP concentration of approximately 1 nM. The AuNP multiplexing solution (20 μL) was added to the MMP−target complexes, and the hybridization was allowed to proceed at 25 °C for 2 h under rotation. The system was washed three times with the assay buffer (200 μL) to remove nonspecifically bound AuNPs. The plasmids were then released from the AuNP probes by the addition of DTT (1 M, 5 μL) in the assay buffer. The reaction was allowed to proceed at 50 °C for 10 min and 25 °C for 50 min under rotation. Introduction of a magnetic field removed all MMP-linked structures, and the rest of the solution was purified free of DTT through a 10 kD centrifuge filter device (15000 rpm, 15 min). The AuNP probe was also removed due to the firm attachment to the filter device. Nanopure water (100 μL) was used to recover the plasmids by first incubation for 10 min and then centrifugation at 3000 rpm for 5 min. The plasmids were then freeze-dried and subjected to *in vitro* expression in the *E. coli* T7 S30 extract system. The peptide tag from the peptide of tandem repeats was obtained by incubating the expression product in cleaving solution (0.5 M hydroxylamine, pH 8.6) at 45 °C for 24 h, and the CM4 peptide was released using the release buffer (0.5 M NaCl, 20 mM Tris, 5% glycerol, 2 mM EDTA, 10 mM DTT, 0.01% Tween 20, pH 7.5) from the fusion protein N^{pro}-EDDIE-CM4 at 25 °C for 24 h. Finally, the target peptides were desalted using ZipTipC18 pipet tips and subjected to MALDI-TOF MS analysis using HCCA as the matrix.

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Supporting Information Available: Characterization of the probes and DNA detection data. This material is available free of charge via the Internet at http://pubs.acs.org.

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